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OMIP-009: Characterization of Antigen-Specific Human T-Cells

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Additional Supporting Information may be found in the online version of this article.

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• **Key terms**
intracellular cytokines; vaccines; T cells; HIV

Purpose And Appropriate Sample Types

The panels described in this article are designed to characterize the immunological response of human T-cells to vaccination by measuring the frequency, phenotype, and function of CD4 and CD8 T-cells. Subsequent qualification of the panel allows for comparison of intra- and inter-laboratory outcomes between different vaccine trials such that those vaccine formulations that reveal a possible correlate of protection against infection may be moved forward in the regulatory process. Although the panel was developed for batch analysis of cryopreserved PBMC samples, the assay may also be performed with fresh cells (Table 1).

Background

Our goal was the development of a standardized multicolor panel that (1) uses stable markers following cryopreservation and stimulation, (2) is amenable for use on com-

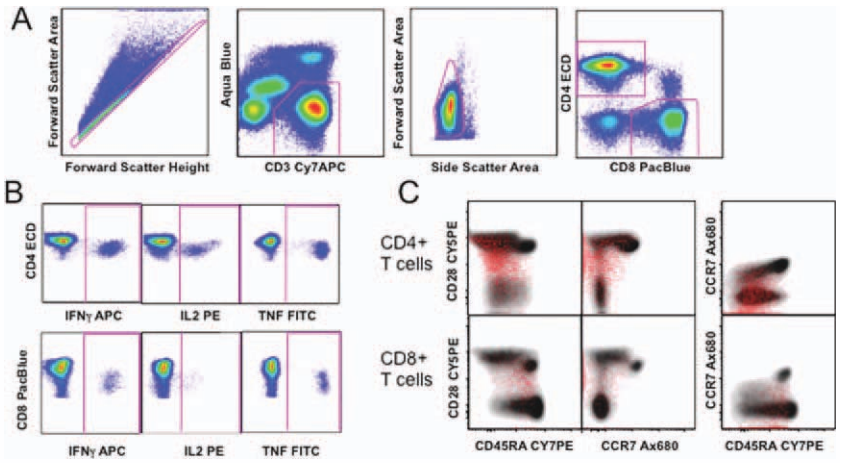

Figure 1. Progressive gating strategy. Data shown are from CMV+ donor cells stimulated with pp65 peptide pool (15-mers overlapping by 11). **A**: Singlets are identified through the use of a FSC-A vs. FSC-H plot. Nonviable and CD3 negative cells are excluded, allowing for the selection of the live CD3+ T-cells only. A FSC-A/SSC-A plot permits the additional removal of very low-scatter cells. The selected CD3+ T cell population is then further delineated into CD4+ and CD8+ T-cells. **B**: Gated on either CD4+ or CD8+ T-cells, the percentage of responding cells for each cytokine is determined. **C**: Using Boolean gating logic in the FlowJo analysis program, the ''or'' function is used to create a single gate of all cytokine-producing cells from a combination of existing cytokine gates, i.e., IFNγ+ or IL2+ or TNF+. Thus any cell that makes one or more cytokines is included in the gate. The total cytokine response (red) is then overlaid onto its respective CD4+ (top) or CD8+ (bottom) T cell lineage (gray) to identify the maturation and activation phenotype of the responding cells.

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Table 1. Summary table for OMIP-009

PURPOSE	FREQUENCY, PHENOTYPE, AND FUNCTION OF CD4+ AND CD8+ T-CELLS
Species	Human
Cell types	Cryopreserved or fresh PBMC
Cross references	Foulds et al. OMIP-005

mercial 10+ color flow cytometers, (3) uses commercial reagents, and (4) can be qualified and/or validated. Accordingly, the authors began designing a basic, robust 7-color staining panel into which they could easily introduce additional immunoprofiling markers for future panels (Fig. 1). The optimization strategy was performed as described (1). As shown in Table 2, marker selection was determined and included identifiers of lineage (CD3, CD4, and CD8), function (IFNγ, IL2, and TNF), differentiation stage (CD45RA, CD28, and CCR7) and viability (Aqua Blue). Because identification of true functional responses from background was the most important aspect of the panel development, the cytokines were the first group of markers optimized by fluorochrome. The majority of commercially available conjugates of IFNγ, IL2, and TNF from five major vendors were obtained for the purpose of titrating, characterizing, and ranking them on the basis of brightness, background, and separation (2). Although included only in the more complex 10-color panel, fluorochromes for the measurement of memory markers CD28 and CD45RA were selected next due to the fewer number of conjugate choices available for this category of antibodies. To aid in the delineation of central memory from transitional memory T-cells, CCR7 was also included. Subsequently, because their performance is well established, the T-cell lineage markers, CD3, CD4, and CD8 were added to the remaining instrument channels. Selected last because of the diversity of available colors, one of eight available amine reactive viability dyes, Aqua Blue, was included in the panel. This dye allows for the exclusion of dead cells that may nonspecifically bind antibodies, thus reducing background (3). Additional developmental strategies including reagent titrations, conjugate ranking example, and staining protocols may be found in the supplemental information on-line.

Table 2. Reagents used for OMIP-009

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
IFNγ	B27	APC	Function
IL-2	MQ1–17H12	PE	
TNF	Mab11	FITC	
CD3	SP342	APC-Cy7	Lineage
CD4	T4	ECD (Texas Red-PE)	
CD8	RPA-T8	PacBlu	
CD45RA	L48	PE-Cy7	Memory/ differentiation
CD28	CD28.2	PE-Cy5	
CCR7	150503	Ax680	
Dead cells	–	AqBlu	

APC, allophycocyanin; AqBlu, LIVE/DEAD fixable aqua blue dead cell stain; Ax, Alexa; Cy, cyanine; ECD, energy coupling dye; FITC, fluorescein isothiocyanate; PacBlu, pacific blue; PE, phycoerythrin.

Similarity To Published OMIPs

None.

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Literature Cited

1. Lamoreaux L, Roederer M, Koup R. Intracellular cytokine optimization and standard operating procedure. Nat Protoc 2006;1:1507–1516.
2. Mahnke Y, Roederer M. Optimizing a multicolor immunophenotyping assay. Clin Lab Med 2007;27:469–485.
3. Perfetto, S, Chattopadhyay P, Lamoreaux L, Nguyen R, Ambrozak D, Koup R, Roderer M. Amine-reactive dyes for dead cell discrimination in fixed samples. Curr Protoc Cytometry 2010 Jul; Chapter 9: Unit 9.34